*International Journal of Odonatology*, 2018
Vol. 21, No. 1, 55–70, https://doi.org/10.1080/13887890.2018.1437478



# Larval development and behavior of *Rhionaeschna marchali* Rambur (Anisoptera: Aeshnidae) under captivity conditions

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(*Received 24 September 2017; final version received 2 February 2018*)

Very little is known about the biology of larval odonates from the Neotropical region, and in particular there are no data on behavioral changes during ontogeny and growth ratios, though both are crucial to understanding the dynamics of Odonata communities. Here we study growth ratio, development patterns and behavior of *Rhionaeschna marchali* larvae. We characterized larval instars using morphometric variables and describe their general behavior. Larvae were obtained from eggs laid by two females in the laboratory. They were maintained in individual containers until their emergence or death. Larvae hatched between 26 and 30 days after laying, and total development time was 340.5 ( ± 5.9) days, with 15 instars. Growth ratios between successive instars averaged 1.12 for head width, 1.25 for head length, 1.20 for antenna length, 1.76 for forewing–pad length, 1.74 for hind wing–pad length, 1.19 for metafemur length and 1.22 for total length. *Rhionaeschna marchali* larvae spent most time "resting" and "grooming". As size increased, larvae became more active and time "resting" decreased. The behavior "upwards abdomen bend" showed a decreasing trend with size, while "body bend downwards" became more common with increasing size. The high altitude (2600 m) of the region acts as a limiting factor for growth, and therefore this species completes one generation per year, similar to many temperate species.

**Keywords:** Odonata; dragonfly; Neotropical region; ontogenetic changes; morphometric variables

## Introduction

Odonates are insects with complex life-cycles, whose longest stage is the larva (Stoks & Córdoba-Aguilar, 2012), which lasts for months or even years. This fact points to the relevance of larval ecology and morphology (e.g. growth ratios) to understanding the dynamics of Odonata communities (Goretti, Ceccagnoli, La Porta, & Di Giovanni, 2001; Hawking & New, 1996), e.g. species with instars whose structures (e.g. legs) vary faster in size could have advantages to compete for food and space, and thus restricting or eliminating other species. High mobility and foraging activity are very important to some species because these traits are associated with more encounters with prey, which may generate a higher growth and development rate (McPeek, 1998). For this reason, an estimation of the ontogenetic changes in captivity conditions is the first step to further research about population dynamics in the field.

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The profile for larval growth ratios varies widely within and between species, but in other cases, could be similar in different instars of the same species exhibiting allometric or isometric growth (reviewed by Corbet, 2002). Thus, growth ratios can vary during ontogeny, showing small or major changes depending of the variables measured and the species (e.g. aeshnid larvae tend to have a high number of instars and slow development), where dimensions with a smooth progression of growth ratios such as head width or metafemur length are useful in determining the larval instar (Corbet, 2002).

Larval growth is affected by the environmental conditions of aquatic habitats, and particularly by the hydroperiod (Johansson & Suhling, 2004), with faster development in temporary habitats. Both larval density and the availability and type of food regulate larval development (Banks & Thompson, 1987; Hassan, 1976). Likewise, temperature and photoperiod (usually related to latitude) are factors influencing larval growth (Corbet, 2004). The larval growth patterns are fundamental to understanding voltinism and seasonal regulation, as well as to allow for the identification of early larval instars (Corbet, 2002). However, the study of larval growth from field data is difficult, due to technical (capture of small larvae) and taxonomic impediments (identification of the smallest larvae). Hence, to unequivocally identify each instar, the growth of the individuals usually has to be monitored under laboratory conditions.

The behavior of larval odonates shows adaptations mainly associated with the breathing process, camouflage and predation (Corbet, 1966). When the oxygen concentration is low, larvae of some species climb to breathe at the water surface, exposing their thoracic spiracles and other body parts, depending on the age of the larva. Apparently, these behaviors are common during pre-emergence in a number of species of Aeshnidae (Corbet, 1966). Adaptations to avoid drying lead the larvae of some species to leave a drying water container and walk in search of a new body of water (Piersanti, Rebora, Salerno, & Gaino, 2007). These situations have important implications in the duration and continuation of the larval development, because low levels of oxygen or water may impede development or increase the time to complete metamorphosis (Corbet, 1966). On the other hand, a common behavior of many odonate larvae is to remain motionless as a mechanism for avoiding predators (Johnson, 1991) or ambushing their prey (Suhling, Sahlen, Kasperski, & Gaedecke, 2005). Successful prey capture also requires antennal and tarsal movements, and head orientation in pursuit of an objective, and finally the ejection of the labium to grasp the prey (Corbet, 2004; Suhling et al., 2005). Apparently, the visual system is of little relevance in early instars, but as larvae grow, their eyes become a powerful organ, allowing the larvae to be highly active in prey capture and predator detection (Corbet, 1966).

The Anisoptera genus *Rhionaeschna* Förster, 1909, comprises 42 species distributed from South Canada to South Argentina (Garrison, von Ellenrieder, & Louton, 2006; von Ellenrieder, 2003), with its highest diversity along the Andes (von Ellenrieder, 2003). The autapomorphy defining the genus is the presence of a conical tubercle bearing denticles on the first abdominal sternum in the imago (von Ellenrieder, 2003). However, unique characters for the larval stage are still unknown (Rodríguez & Molineri, 2014). *Rhionaeschna marchali* (Rambur, 1842) is a South American dragonfly, with a distribution ranging from Bolivia to north Venezuela; the adults are large (body length 56–63.5mm) and characterized by a thorax with yellow mesepimeral and metepimeral stripes and a pale reddish brown abdomen with light blue and yellow spots (von Ellenrieder, 2003). It is a territorial species found in crop pastures, streams and ponds with muddy bottoms surrounded by *Juncus* spp. (Juncaceae) and *Typha* spp. (Typhaceae) (Campos, 1922; Limongi, 1983). Limongi (1983) described the larva by supposition based on exuviae collected at temporary pools in Venezuelan forests. He characterized the brown larva as medium sized (34–37mm), with the wing pads extending to posterior margin of abdominal segment 4, and the lateral spines of abdominal segments 7 and 8 shorter than the lateral spines of segment 9. The biology of *R. marchali* is poorly known, and there is no particular information available about

larval behavior and development. In this paper, we characterize the larval instars of *R. marchali* using morphometric variables and describe its general behavior.

## Material and methods

### *Study area and laboratory breeding*

The fieldwork was carried out in the Tominé impoundment, Guatavita municipality, at 4.933°N, 73.50°W and an elevation of 2600 m asl, in the Cundinamarca department, Colombia. The predominant vegetation in this area is *Acacia* sp. (Fabaceae), *Commelina* sp. (Commelinaceae), *Eichhornia crassipes* (Pontederiaceae), and *Brachiaria* sp. (Poaceae).

In order to obtain eggs, two adult females were collected on 11 March and 4 June 2013. In the laboratory, these females oviposited on moist filter paper (Cordero-Rivera, 1990), and the eggs and larvae were maintained in water at an ambient temperature ($\sim$4–12°C). The eggs were checked daily until hatching. Larvae were individually placed in numbered plastic containers (295 ml), which were filled with fresh water from the collecting location. In each container, a wooden stick was provided as support for the emergence. To maintain the water level, dechlorinated water was added each week. The larvae were fed daily *ad libitum* on *Daphnia* Müller, 1785 (Daphniidae), *Eucyclops* Claus, 1893 (Cyclopidae), *Artemia* Leach, 1819 (Artemiidae), larvae and adults of *Drosophila melanogaster* Meigen, 1830 (Drosophilidae), adults of hemipterans (Corixidae, Notonectidae, and Myridae), and dipterans (Calliphoridae and Muscidae).

### *Analysis of larval development*

After each molt or death, exuviae or larvae were preserved in 70% ethanol. In order to analyze the larval development, several morphological characteristics were measured (supplemental material Tables S1, S2), with a micrometer attached to a 20 × zoom binocular microscope, providing an accuracy of 0.1 mm. We measured larval head width (including eyes), which is a widely used variable to describe size of odonate larvae (Corbet, 2004). Likewise, we measured larval head length, metathoracic femur length, length and width of labrum, antenna length, number of antennal segments, wing pad length, and total length. Boxplot graphics were used to describe growth patterns for each structure and to analyze tendencies related to sex. Linear regressions between pairs of variables were calculated to study their relation and the existence of allometric growth. Also, combinations of variables were used to generate scatter plots, and to determine which of them separates larval instars partially or completely. Prementum measurements were excluded from all analyses, due to a strong correlation with those of the head ($R^2 = 0.99$, $p < 0.001$).

Instars were counted using the terminology of Corbet (2004): F-0 (final instar), F-1 (penultimate instar), etc. Larvae of instars F-14 and F-13 could not be sexed, due to the absence of reliable morphological characters. According to Dyar (1890), we calculated the mean growth ratio between instars using the following expression: $F_{(n+1)}/F_{(n)}$, where $F_n$ is the value of each variable at instar n, and $F_{(n+1)}$ is the value of the variable at a subsequent instar. We used two ways to calculate life cycle length. First, we averaged the time (in days) spent by all surviving individuals on each instar (egg, prolarva, larva and adult) (Table 1). The sum of those means was considered equal to the total life cycle length. Second, we averaged the time spent in each instar by the four individuals that were kept alive until emergence (Table 1).

### *Behavioral observations*

Following the focal animal temporal sampling technique (Altmann, 1974), each observation session (from 08:00 to 17:00 h COT (UTC-5)) was divided into periods of 10 min and the behavior

Table 1. Duration of each stage of development and larval instar of *R. marchali*.

| Stage/instar | N | Min | Max | Mean | Standard deviation |
|---|---|---|---|---|---|
| Egg | 52 | 26 | 30 | 27.8 | 1.37 |
| F-14 | 52 | 11 | 16 | 13.5 | 1.69 |
| F-13 | 47 | 21 | 27 | 24.1 | 1.71 |
| F-12 | 46 | 16 | 25 | 21.3 | 2.13 |
| F-11 | 46 | 15 | 26 | 20.0 | 1.56 |
| F-10 | 45 | 17 | 25 | 20.7 | 1.91 |
| F-9 | 44 | 16 | 24 | 20.4 | 1.63 |
| F-8 | 43 | 18 | 25 | 21.3 | 1.58 |
| F-7 | 37 | 17 | 23 | 20.2 | 1.12 |
| F-6 | 31 | 21 | 25 | 22.8 | 1.31 |
| F-5 | 29 | 17 | 24 | 20.5 | 1.55 |
| F-4 | 26 | 17 | 25 | 21.5 | 1.81 |
| F-3 | 25 | 16 | 25 | 21.3 | 2.09 |
| F-2 | 24 | 19 | 23 | 20.3 | 0.86 |
| F-1 | 20 | 14 | 26 | 21.2 | 2.62 |
| F-0 | 20 | 15 | 26 | 20.0 | 2.57 |
| Adult | 4 | 4 | 6 | 5.0 | 0.81 |
| **Total duration (all individuals)** | | | | **342.6** | |
| **Total duration (4 surviving individuals)** | 4 | 334 | 346 | **340.5** | 5.91 |

Table 2. List of behaviors studied in *Rhionaeschna marchali* larvae.

| Behavior | Name | Explanation |
|---|---|---|
| B1 | Rest | The larva remains motionless for long periods, usually concealed below plants or detritus. |
| B2 | Upwards abdomen bend | The larva bends up (at an angle $\leq 80°$) the apex of the abdomen repeatedly (three or more times), alternating vertical and horizontal positions. |
| B3 | Lateral abdomen bend | The larva bends the abdomen laterally, from the fourth segment. |
| B4 | Grooming with forelegs | The larva grooms parts of the head, especially the eyes and the antennae, using the forelegs. |
| B5 | Movements of forelegs | The larva rubs its forelegs, while firmly stands on the other legs. |
| B6 | Fore- and median leg rubbing | The larva rubs an anterior leg with a medium leg of the same side, while firmly stands on the other legs. |
| B7 | Repeated movement of a median leg while consuming a prey | While consuming a prey, the larva remains on five legs, raising one of its middle legs, which moves up and down several times. |
| B8 | Body bend downwards | The larva tilts its head and the abdominal apex downwards, creating a convex curve in the dorsal region of the abdomen (S3–S6). |
| B9 | Body bend and tapping the container | From position B8, the larva straight strongly and simultaneously hits the container with the lateral region of the body. |
| B10 | Sit-and-wait prey capture | The larva remains motionless and only catches prey that pass close to the front of it. |
| B11 | Head orientation to prey | The larva locates the prey and carefully directs its head towards where it is located, then, carefully and slowly directs the rest of its body towards it to attack it. |
| B12 | Prey pursuing | The larva locates the prey, pursues actively and attacks it, sometimes needing several attempts to capture it. |

of each larva placed in a different numbered plastic container was observed and recorded. A matrix of the observations for each larva, including date, time, and code number of the plastic container, was tabulated. The behaviors observed in *R. marchali* larvae, were classified into 12 categories associated with preying and other activities (Table 2, Figure 1). To avoid influencing larval behavior, observations were made from a distance greater than 20 cm (F-14 to F-11) or 120 cm (F-10 to F-0) from the container, without making sudden movements or loud sounds.

Two types of tests were used to determine if there were behavioral differences between instars. In the case of behaviors assessed in a quantitative form (B1 to B8), two-sample *t*-tests,

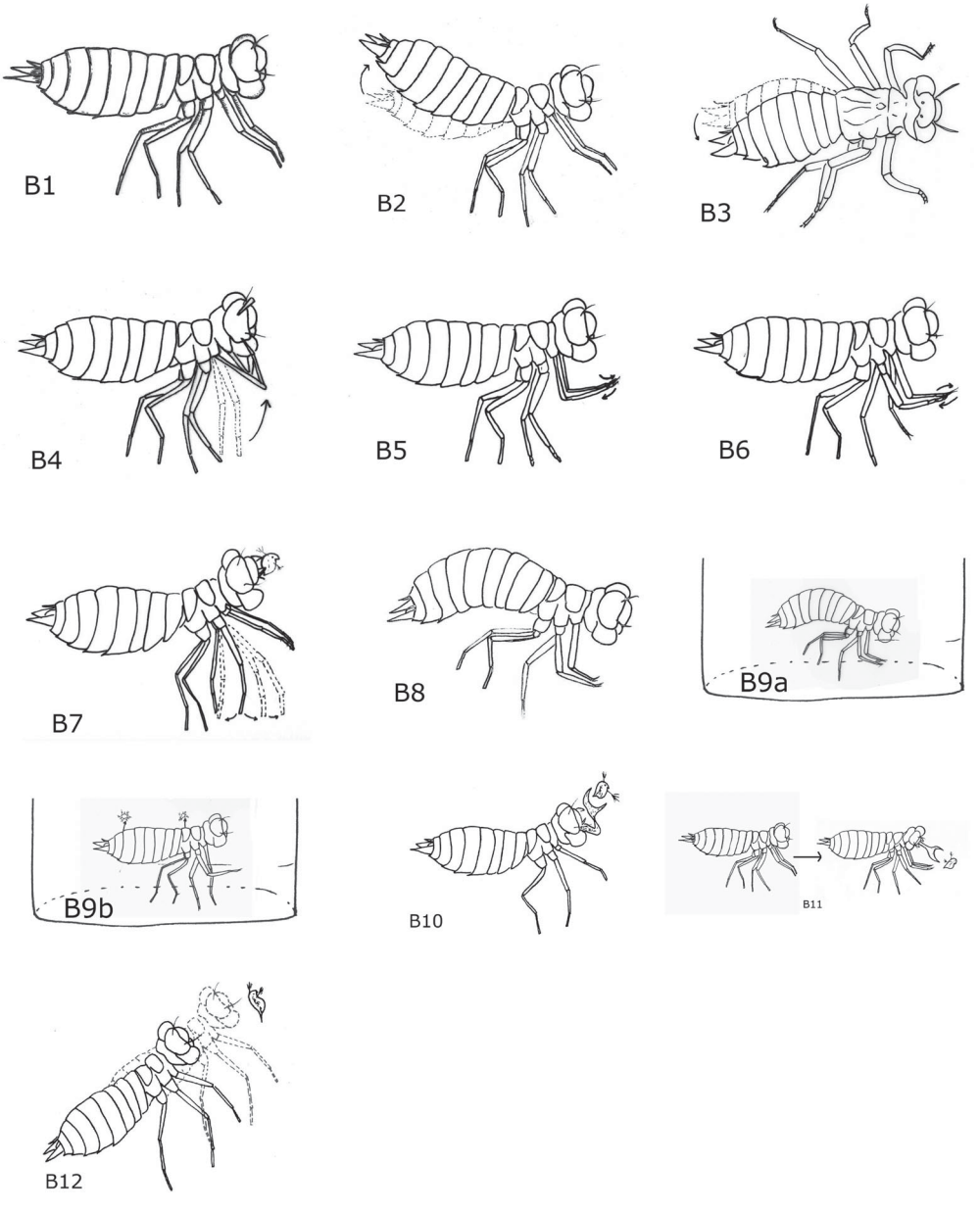


Figure 1. Behaviors of *Rhionaeschna marchali*; see explanation in Table 2. Illustrations: P. Camacho.

Mann–Whitney U and Kruskal–Wallis tests, were used depending on the number of groups (instars exhibiting each behavior) and normality of data. Behaviors described with qualitative variables (B10 and B11, Table 2), were analyzed by homogeneity contrast tests of independent samples and dichotomous variables with Yates correction (when necessary). In the case of behaviors B9 and B12, there was no variability between individuals.

Normality, Kruskal–Wallis, Mann–Whitney test and scatter plots were done in Past 2.16 (Hammer, Harper, & Ryan, 2001), and linear regressions, homogeneity contrasts and boxplot figures with R Wizard 1.1 (Guisande, Vaamonde, & Barreiro, 2014).

## Results

### *Larval development*

The first female laid 400 eggs (oviposition lasted 25 minutes), and 112 (28%) of them hatched, while the second female laid 46 eggs (oviposition lasted nine minutes), and 24 (52%) hatched. Hatching of larvae occurred between 26 and 30 days after laying. The eggs were maintained for six additional months, but no additional larvae hatched. Overall, 192 *R. marchali* individuals were analyzed, with 112 individuals used for behavioral observations (Table 3), 22 individuals used for measurements (Table 3) and 52 individuals used for determining the duration of each instar (Table 1). The average life cycle was calculated as 342.6 days including all individuals and 340.5 (SD 5.9, Table 1) considering only the four that completed the whole cycle (only four individuals emerged to adults, and all reached 15 instars), F-13 was the longest instar, and F-14 was the shortest in duration (see Table 1 for detailed data on the duration of each instar). The life cycle was completed after 15 instars including the prolarva. Larval instars lasted, on average, 20.1 days (Table 1). Scatter plots showed that head width/head length and head width/total length variables were the best for separating instars (Figure 2a–e). Head width/antenna length, head width/forewing–pad length and head width/hind wing–pad length were useful in separating most instars, except for F-2 and F-3. All structures, except wing-pads, showed continuous growth from F-14 to F-1. Wing pads developed at F-12 (Figure 3). Initially we considered the prolarva as a different instar, but we could not separate this instar from F-14 (no significant differences were found; data not shown), so that they might represent the same instar. When plotted against time, larvae showed an acceleration in growth in all measured structures between F-3:F-2, F-2:F-1 and F-1:F-0 (Figure 4), compared to other instars.

Growth ratios between successive instars ranged between the following values: head width, 1.06–1.61 (mean 1.12); head length, 0.88–1.69 (mean 1.25); antenna length, 1.00–1.9 (mean 1.20); forewing–pad length, 0.99–4.75 (mean 1.76); hind wing–pad length, 1.02–4.53 (mean

Table 3. Behavior of *R. marchali* larvae.

| Instar | N | E | %m | %f | Behavior | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | B1 min | B2 s | B3 s | B4 s | B5 s | B6 s | B7 s | B8 min | B9 % | B10 % | B11 % | B12 % |
| Egg | 36 | 0.0 | – | – | – | – | – | – | – | – | – | – | – | – | – | – |
| F-14 | 112 | 20 | – | – | 9.7 | 0.5 | 0 | 0 | 3.0 | 0 | 0 | 0 | 0 | 100 | 0 | 0 |
| F-13 | 63 | 5 | – | – | 8.4 | 0.5 | 0 | 0 | 1.2 | 0 | 0 | 0 | 0 | 74.0 | 0 | 0 |
| F-12 | 53 | 18 | 5.5 | 94.5 | 8.4 | 0.3 | 0.3 | 51 | 0 | 5 | 5 | 0 | 0 | 88.0 | 100 | 0 |
| F-11 | 52 | 9 | 0.0 | 100 | 8.4 | 0.3 | 0.1 | 48 | 0 | 5 | 5 | 0 | 29 | 90.3 | 100 | 0 |
| F-10 | 46 | 18 | 5.5 | 94.5 | 8.1 | 0.2 | 0 | 54 | 0 | 0 | 0 | 0 | 26 | 67.4 | 100 | 0 |
| F-9 | 42 | 22 | 18.1 | 81.9 | 7.6 | 0.2 | 0 | 54 | 0 | 0 | 0 | 0 | 0 | 100 | 100 | 0 |
| F-8 | 36 | 17 | 29.4 | 70.6 | 7.6 | 0.2 | 0 | 25 | 0 | 0 | 0 | 0 | 0 | 91.6 | 100 | 0 |
| F-7 | 30 | 13 | 23.0 | 67 | 7.6 | 0.2 | 0 | 33 | 0 | 0 | 0 | 0 | 0 | 70.0 | 100 | 0 |
| F-6 | 20 | 8 | 62.5 | 37.5 | 7.6 | 0.2 | 0 | 21 | 0 | 0 | 0 | 5.6 | 0 | 65.0 | 100 | 0 |
| F-5 | 20 | 8 | 25.0 | 75 | 7.4 | 0.2 | 0 | 22 | 0 | 0 | 0 | 5.8 | 0 | 45.0 | 100 | 0 |
| F-4 | 20 | 15 | 53.3 | 46.7 | 7.4 | 0.1 | 0 | 22 | 0 | 0 | 0 | 6.4 | 0 | 65.0 | 100 | 0 |
| F-3 | 20 | 6 | 33.3 | 66.7 | 7.4 | 0.1 | 0 | 21 | 0 | 0 | 0 | 6.4 | 0 | 75.0 | 100 | 0 |
| F-2 | 12 | 5 | 80.0 | 20 | 7.3 | 0 | 0 | 24 | 0 | 0 | 0 | 6.6 | 0 | 0 | 100 | 100 |
| F-1 | 8 | 7 | 42.8 | 57.2 | 7.2 | 0 | 0 | 21 | 0 | 0 | 0 | 7.1 | 0 | 0 | 50.0 | 100 |
| F-0 | 4 | 21 | 42.8 | 57.2 | 7.2 | 0 | 0 | 24 | 0 | 0 | 0 | 7.2 | 0 | 0 | 100 | 100 |
| Adult | 4 | 0 | 0.0 | 100 | – | – | – | – | – | – | – | – | – | – | – | – |

Notes: N, number of individuals; E, number of exuviae measured; %m, percentage of males from exuviae measured; %f, percentage of females from exuviae measured; B1, rest; B2, upwards abdomen bend; B3, lateral abdomen bend; B4, grooming with forelegs; B5, movements of forelegs; B6, fore- and median leg rubbing; B7, repeated movement of a median leg while consuming a prey; B8, body bend downwards; B9, body bend and tapping the container; B10, sit-and-wait prey capture; B11, head orientation to prey; B12, prey pursuing.

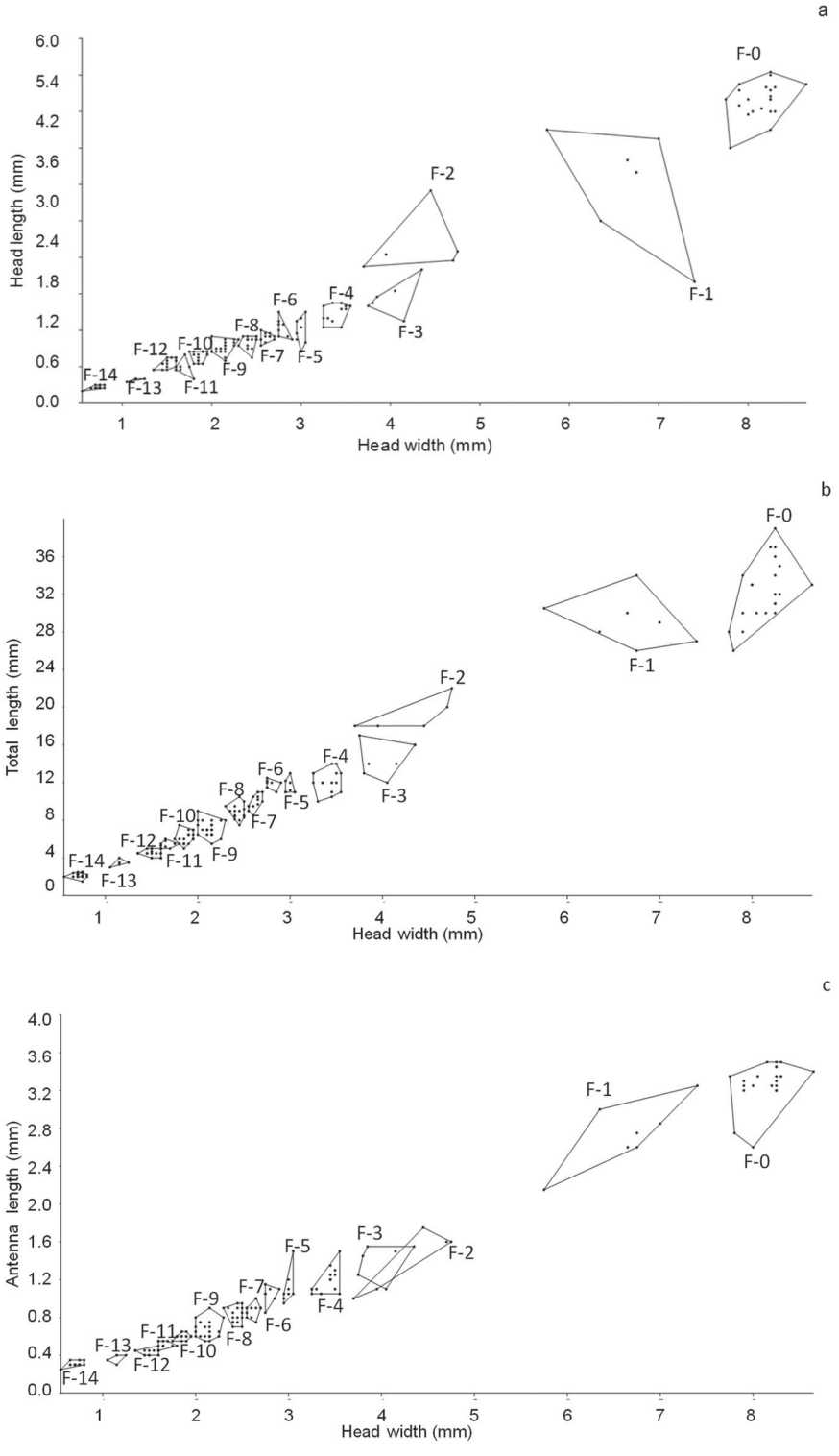


Figure 2. Scatterplots using pairs of variables measured in *R. marchali* larvae.

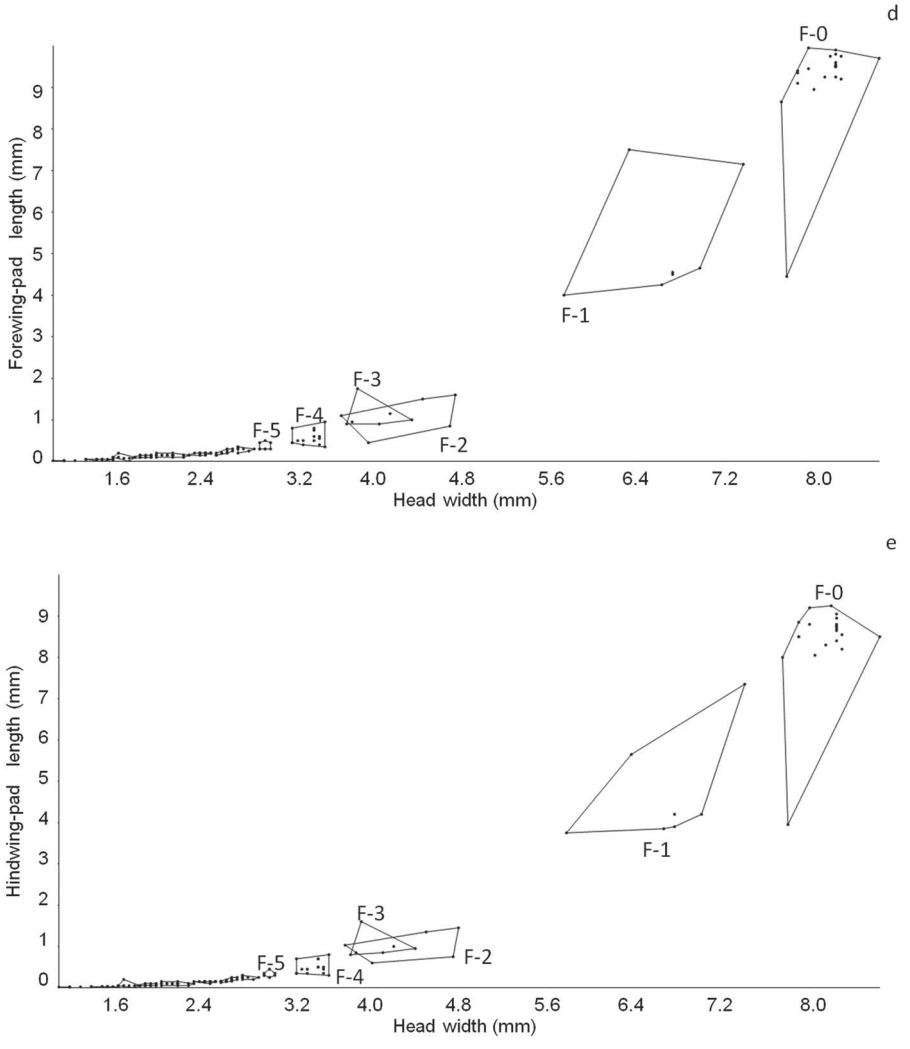


Figure 2. Continued.

1.74); metafemur length 1.01–1.45 (mean 1.194) and total length 0.97–1.60 (mean 1.22). Growth ratios of fore- and hind wing pads were higher than those of the other biometric characteristics measured, confirming that they show some positive allometry in comparison with the other variables. The highest growth rate was found from the first to the second instar (F-14 to F-13). Sex ratio varied over the development, with a higher male proportion in F-6, F-4 and F-2 and more females in the remaining instars (Table 3). There were no size trends related to sex in the measured variables (Figure 5a–c).

### *Behavioral study*

*Rhionaeschna marchali* larvae spent most of their time "resting" and "grooming" (Table 3). As size increased, larvae became more active and time "resting" decreased from 9.7 min (instar F-14) to 7 min (F-9 to F-1). The "upwards abdomen bend" behavior (B2, Tables 2 and 3) also showed a decreasing trend with size. "Body bend downwards" (B8) appeared in instar F-6 and

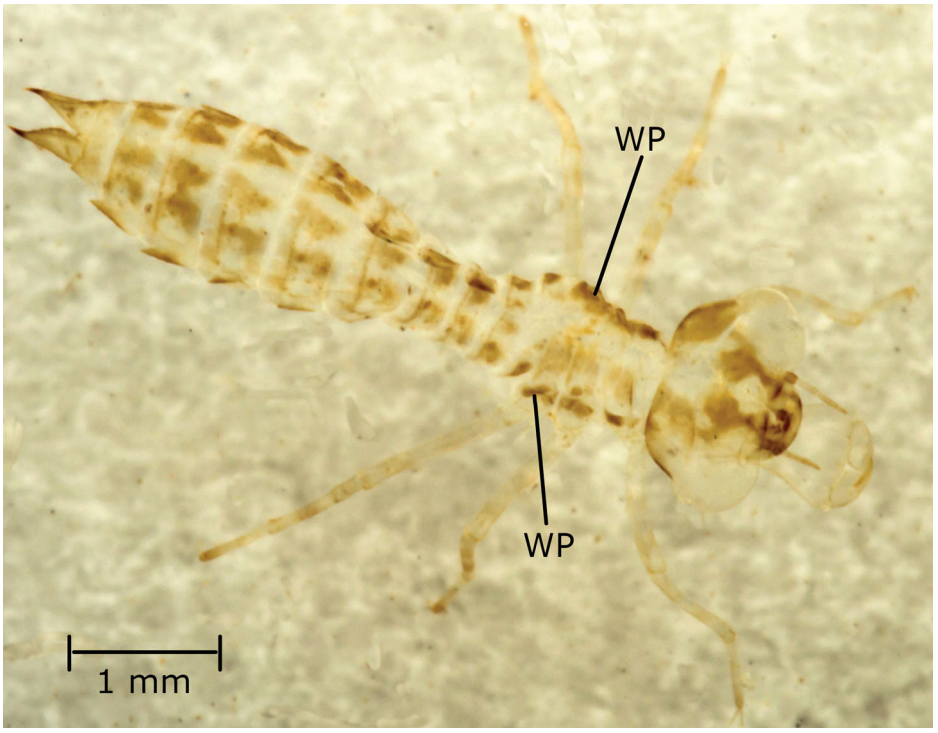


Figure 3. Wing pads in third larval instar of *R. marchali*. WP: wing pad. Photo: S. Alvarez.

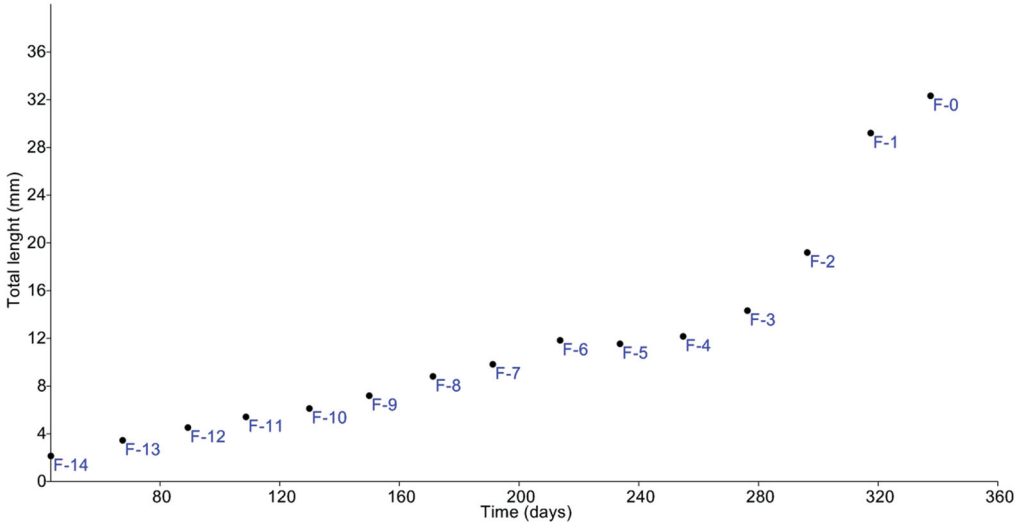


Figure 4. Development of *R. marchali* larvae over time, using total length as proxy of growth.

became more common with increasing size, increasing from 5 min in F-6 to 7 min in F-1. Time spent "grooming with forewings" (B4, 21–54s) was highly variable among instars. Other behaviors were observed in just a few individuals and could not be analyzed (Table 3).

A common behavior was to "sit-and-wait," hunting only those prey which passed very close (B10, Figure 1). This strategy was observed in 45–100% of individuals in instars F-14 to F-3,

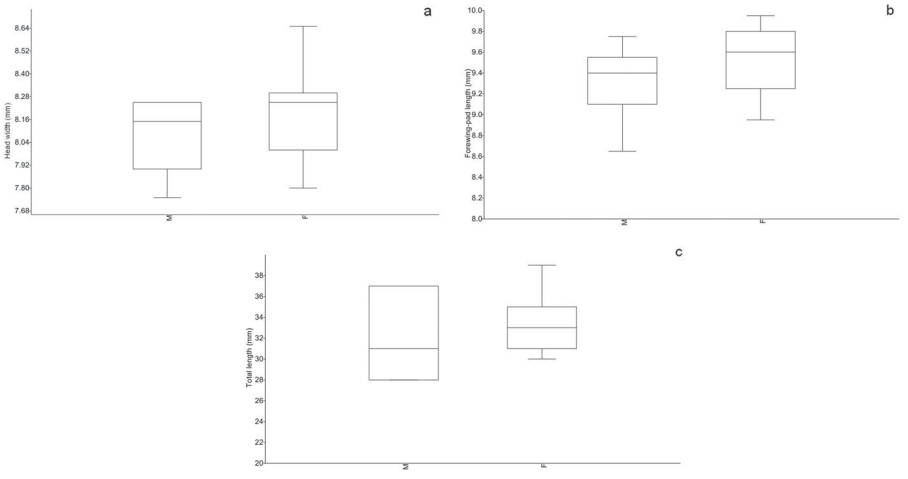


Figure 5. Boxplots of some morphological variables in *R. marchali* F-0 larvae. F: female, M: male.

and disappeared in larger instars. A second hunting strategy consisted of head orientation (B11, Figure 1) sometimes followed by actively pursuing prey, particularly in the larger individuals. Four larvae (two F-10 individuals and one F-9 and F-8) disappeared from their container. Three of them were found alive in dark corners of the laboratory 5 h, 2 days and 15 days later. These larvae were returned to the containers, but none of them reached the last instar. The day before and after each molting *R. marchali* larvae did not feed, remaining motionless and most of them even moved away when they noticed some prey close.

## Discussion

### *Life cycle duration*

To our knowledge, there are no studies concerning the life cycle of *R. marchali*, and a few studies refer only to the final instars (e.g. Limongi, 1983; von Ellenrieder, 2003). The *R. marchali* life cycle took a little longer than 11 months, but it is difficult to make comparisons with any other species of the genus because the information in this topic is limited. The only reference known to us is the length of *Rhionaeschna mutata* (Hagen, 1861) life cycle, which takes between 36 and 48 months (Nikula, Ryan, & Burne, 2007). For other aeshnid species, data vary from three to 48 months (reviewed by Corbet, Suhling, & Soendgerath, 2006).

This variation in the duration of the life cycle has been associated to the local conditions of the habitat in which the organism develop (Corbet, 1966). The main variables that influence larval growth under natural conditions are photoperiod, temperature, seasonal regulation and the availability of food (Corbet, 2004; Harrison, Woods, & Roberts, 2012; Pritchard, Harder, Kortello, & Krishnaraj, 2000). In our study, these conditions were controlled and the temperature was in the same range (4–12°C) throughout the study. The availability of food however, could be the condition more relevant to rear *R. marchali*, since larvae were fed *ad libitum* in an environment without parasites or predators, generating an ideal environment for larval development. Our findings are consistent with several researchers, who have found that rapid development and high growth rates are associated with a high rate of encountering prey providing higher food intake (Johansson, 2000; Wissinger, Whiteman, Sparks, Rouse, & Brown, 1999).

Other variables influencing larval development, include the origin of individuals, that is, if they come from resident or non-resident populations (Trottier, 1971), their genetic constitution (Stoks, Geerts, & De Meester, 2014), and the abundance of predators or presence of parasites (McPeek, 2008), ambient temperature and seasonal regulation (J. Abbott pers. comm). The controlled conditions in our study allow us to assume that the females from which eggs were obtained came from a resident population under similar conditions to those used to rear the larvae.

The number of eggs laid by aeshnid females is reported with a range between 100 and 5200 (e.g. 243 in *Aeschna isoceles* (Müller, 1767); Corbet, 1966; see also Koch, 2015a). One of our females apparently laid a large clutch (400 eggs), but the other one only laid a partial clutch. Waage (1978) showed that in endophytic ovipositing species, the quantity of eggs laid is affected by the morphology of the plant species, as well as its resistance to the ovipositor. Oviposition selectivity of many species is so strong, that many do not lay in artificial substrates. On the other hand, it is possible that the female that laid fewer eggs had already deposited most of her clutch before she was captured (K. Tennessen pers. comm). We did not change water during the first 30 days, and this could have affected embryo survival, because anoxic conditions contribute to Odonata death in their aquatic stages (Van Gossum, Sánchez-Guillén, & Cordero-Rivera, 2003).

Eggs and larvae were exposed to cool temperatures (4–12°C), in which many larvae hatched and survived, however, odonate eggs do not develop below 10°C (Koch, 2015b; Pritchard, Harder, & Mutch, 1996; Rotvit & Jacobsen, 2013), since egg development, growth and activity require optimal temperatures above 20°C (Pritchard et al., 1996; Pritchard & Leggott, 1987). Thus, for *R. marchali*, the hatching and larval development must have occurred between 10°C and 12°C. This temperature, although low, would make it possible for larval development to occur as has been reported for other Odonata species (see Suhling, Suhling, & Ritcher, 2015).

Usually, field-collected larvae cannot be unambiguously assigned to a particular instar of development; however, this can be determined when larvae are reared under controlled conditions, where each molt can be counted (Tennessen, 2017). Nevertheless, even under laboratory conditions, it has been reported that the number of molts between hatching and metamorphosis to the adult stage varies from nine to 17, with different values for the same species (Corbet, 2002; Sternberg, 1995). In *R. marchali*, the overall number of instars was similar to the values reported for other aeshnids like *Aeshna cyanea* (12 instars, Goretti et al., 2001), *Anax junius* (13 instars, Calvert, 1934), *Hemianax papuensis* Burmeister, 1839 (15 instars, Rowe, 1991) and *Anax imperator* Leach, 1815 (17 instars, Lamelas-López, Florencio, Borges, & Cordero-Rivera, 2016). In *R. marchali*, F-14 was the shortest instar and showed the highest death rate (43.7%), possibly due to the inability of larvae to grasp small prey (e.g. *Artemia*).

For successful prey capture, eyes are essential in larvae in the last instar, though, for many species, the antennae and tarsi seem to be crucial in the first instars (Corbet, 1966). In *A. imperator* the eyes are significantly more complex and efficient than in the second larval instar, provided there is a good lighting (Corbet, 1966). This might not be the case for *R. marchali* because many first instar larvae (F-14 to F-12) failed to capture prey under illumination of $\sim$300 lux. This was because, as in most Anisoptera (but not all aeshnids), in the first instar their eyes have too few ommatidia (Ando, 1957). Despite having prey close by, these were not perceived by larvae and in some cases the smallest larvae were frightened and escaped from their potential prey. To increase the success of these small larvae feeding, future breeding experiments should use smaller individual containers.

### *Instar differentiation and growth ratios*

We confirmed that head width serves to detect discrete discontinuities and we agree with other authors in favoring the use of a method based on correlation between pairs of morphological

variables as a relevant tool, especially for the final instar of development (Goretti et al., 2001; Lamelas-López et al., 2016; Palacino-Rodríguez, Sarmiento, & González-Soriano, 2015). For example, Tennessen (2017) recently suggested a method to easily recognize the last instar of Odonata larvae based on the hind wing sheath length/maximum head width ratio, variable which was used to separate the last instar in aeshnids like *Anax junius*, *Boyeria vinosa* (Say, 1839) and *Nasiaeschna pentacantha* (Rambur, 1842). Similar findings were found by Lamelas-López et al. (2016) for *Sympetrum fonscolombii* (Rambur, 1842) and by us for F-7 to F-10 instars but with overlapping in F-3 and F-2. In addition, in the present research, the ratios of head to width/head length (Figure 2a) and head to width/total length (Figure 2b) were the best to separate the 15 instars of *R. marchali*. Due to the development of active hunting strategies as the organism grows, it is possible that head and body size growth (especially the eyes) are more marked in each instar, increasing the organism's ability to locate more accurately its prey, and at the same time to move faster. Early onset (F-12) development of wing-pads in *R. marchali* contrasts with general reports for other odonate species (instar 5–7, Corbet, 2004) and certainly, aeshnid reports from F-8 to F-3 (Assis, Carvalho, & Dorvillé, 2000; Goretti et al., 2001; Lamelas-López et al., 2016; Rowe, 1991). Despite their early onset, the maximum size for this structure does fit the pattern present in other species like *Hemianax papuensis* (Rowe, 1991) or *Boyeria irene* (Ferreras-Romero, 1997) where wing-pads reach only the first four abdominal segments.

Although sexual size dimorphism is a widespread phenomenon in animals (Fairbairn, 1997, 2007), F-0 *R. marchali* larvae did not show sex-biased dimorphism (Figure 5a–c), suggesting that our findings are not in agreement with the overall trend of adult Odonata, where most species exhibit male-biased size dimorphism (Stillwell, Blanckenhorn, Teder, Davidowitz, & Fox, 2010), but neither study reports female-biased size dimorphism in the larval stage (Stoks, 1999). It has however, been reported that size differences between sexes in larval instars do not always lead to the same pattern in adults and that monomorphic larvae could lead to dimorphic adults (Serrano-Meneses, Azpilicueta-Amorin, Szekely, & Córdoba-Aguilar, 2007).

The sex ratio was significantly biased toward females (2.5:1), but the instar average length did not show any tendency between sexes. Although sex ratio of Odonata can differ greatly among taxa or even within a species (Corbet & Hoess, 1998), it often exhibits a slight excess of females in the Anisoptera (Corbet, 2004; Corbet & Hoess, 1998; Cordero-Rivera & Stoks, 2008). Adult populations nevertheless have a male-biased sex-ratio in most studies (Cordero-Rivera & Stoks, 2008). Many species show widely varying sex ratios between sites and years suggesting that environmental factors influence determination of sex (Corbet & Hoess, 1998). However, it is unclear how the factors act. In addition, these apparent male biased populations are likely explained by data that have been preferentially recorded at ponds where males guard territories, but females typically avoid these habitats except during mating.

The highest growth rates for most structures measured in *R. marchali* were found in the first molt, and similar patterns were reported by Corbet (1957) for *A. imperator*. Structures like wing-pads revealed a higher growth rate than other structures (Tables S1, S2), a pattern that matches with the findings of Calvert (1934) for the genus *Anax* and by Goretti et al. (2001) for *Aeshna cyanea*. Comparing *R. marchali* growth rates with other aeshnids and some libellulids, there is no clear difference between them (see Lamelas-López et al., 2016), which may indicate some constraints on growth between successive instars at the level of Odonata. In this order, the lowest growth rates are associated with species such as *R. marchali*, which are adapted to permanent environments, where the temperature and food availability are the main factors affecting growth (Suhling et al., 2015). The air temperatures under which *R. marchali* larvae were grown are within the range of $T_{min}$ (8–12°C) and $T_{max}$ ($\geq$ 35°C) proposed as critical for Odonata growth (reviewed by Suhling et al., 2015). At the same time, it is well known that growth rate depends not only on the quantity and type of available food, but is also influenced by ingestion rates and

intake of nutrients for each species (Culler, McPeek, & Ayres, 2014), where a high amount of food intake can support rapid growth (Pickup & Thompson, 1990).

### *Larval behavior*

Behavioral modifications to avoid predation include reduced activity (moving less frequently or more slowly; reviewed by Stoks, McPeek, & Mitchell, 2003; Suhling et al., 2005). Given that larvae of this species possess no big spines or similar defense structures, there is a functional trade-off (DeWitt, Robinson, & Wilson, 2000) where the lack of anti-predator structures is compensated with an extensive inactivity behavior that reduces the probability of encounter with predators. This adaptive potential would explain an increased activity in larvae in an environment where they perceived they were not attacked.

Abdominal movements of odonate larvae have been named in a general way as "wave abdomen" (Richardson & Baker, 1996). Several hypotheses have been proposed to explain these movements: as an aeration mechanism (Lawton, 1971; Miller, 1994), as warning signals to conspecifics (Rowe, 1985), as movements to effectively process food (Richardson & Anholt, 1995; Richardson & Baker, 1996), an indicator of liberation of metabolites just before molting, or specific movements to accelerate the disposal of the old exoskeleton (Richardson & Baker, 1996). In our study, wave abdomen was related to food consumption (30%) and ecdysis process (70%).

As in other insects, the dragonfly larva uses its legs to walk, swim and climb. However, aeshnid larvae are essentially walkers, where the insect's body can at any point in time form a tripod controlled by the higher centers of the brain (Sviderskii, Plotnikova, Gorelkin, Severina, Yu, & Isavnina, 2014). This walker function was corroborated in B4-B7 behavior, in which larvae sustained their bodies using only some of their legs. Traditionally, leg movements, especially the more sensitive forelegs, have been related to improved tactile detection of prey (Pritchard, 1964), removing particles (e.g. debris, algae), parasites (e.g. mites) and phoretic organisms like protozoa, which interfere primarily with their eyes and their antennae (Leung, Forbes, & Baker, 1999). In other dragonflies, grooming behavior tends to rise substantially when the larva is close to emergence (Leung et al., 1999), but it is reduced in the first instar to avoid sudden movements where the larva has to deal with predators (Baker & Smith, 1997). *Rhionaeschna marchali* larvae generally groomed their eyes and antennae (B4) and the grooming activity decreased as the emergence approaches, coinciding with greater larval endeavors to foraging activities.

*Rhionaeschna marchali* larvae show various foraging modes recorded for Odonata larvae (active, sedentary, visual, and tactile; Corbet, 2004; Sherk, 1977). During the first instars, larvae react slowly to the presence of prey, and their prey-capture patterns were less coordinated than those of last instars (Caillere, 1974; Richard, 1961). It is well known that during the first instars the eyes are not highly developed and the muscle of the labium precludes achieving its optimal length (Caillere, 1972), which as a result creates problems in trapping and consuming prey. For *R. marchali*, in F-12 and F-13, tactile capacity started fading to make way for the use of its eyes, expressed by head, thorax, legs and general body movements that made full body relocation pointing to prey. In later instars, movements and relocation of larvae were more rapid and accurate, with active persecution of prey. According to Rowe (1994), this versatility depends on larvae being able to differentiate types of prey and is in line with their morphology and behavior. In the present study, prey size was gradually increased as larvae were growing up, thus in the first instar, prey were smaller (e.g. *Daphnia*), up to bigger prey in the last instar (e.g. Calliphoridae adults). Until data are available to further investigate this, we conclude that versatility in hunting strategies was related to size and movement speed of prey, with active strategies required by bigger prey, largely held due to an increase in *R. marchali* larvae vision.

## Acknowledgments

We are grateful to Paola Camacho for providing the beautiful illustrations, and Santiago Alvarez for the photo in Figure 3. We also thank Ken Tennessen, John C. Abbott and an anonymous reviewer for the comments that helped us improve this manuscript. We thank Grupo de Investigación en Odonatos y otros artrópodos de Colombia (GINOCO), Grupo de Investigación en Biología (GRIB) of the Biology Department at Universidad El Bosque, and the Centro de Investigación en Acarología for their academic support.

## Funding

AC-R was funded by a grant from the Spanish Ministry with competences in Science, including FEDER funds [CGL2014-53140-P].

## Supplemental data

Supplemental data for this article can be accessed at https://doi.org/10.1080/13887890.2018.1437478.

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